

Agent for the photodynamic diagnosis and therapy of malignant tumours**Description**

The invention concerns an agent for the photodynamic diagnosis and therapy of oncological diseases based on chlorin E₆ compounds and new medical uses of chlorin E₆ compounds.

An agent is described in the Russian patent No. 2152790 for the photodynamic diagnosis and therapy of oncological diseases which is composed of 40 to 90 % by weight chlorin E₆ and 60 to 10 % by weight polyvinylpyrrolidone.

Photosensitizers are necessary for photodynamic cancer therapy. These sensitizers are injected and accumulate mainly in the cells affected by the cancer. The production of cytostatic substances is induced in the cancer cells which contain the photosensitizer by the targeted action of laser light of a certain wavelength. This results in a tumour necrosis.

Known sensitizers for this purpose are haematoporphyrins, phthalocyanines and naphthalocyanines. In practice sensitizers based on porphyrin have proven successful due to their low phototoxicity and suitable sensitivity to the lasers that come into consideration for use.

The combination preparation defined above consisting of a complex of chlorin E₆ and polyvinylpyrrolidone (PVP) has proven to be particularly suitable. Chlorin E₆ has intensive adsorption bands in the spectral range of 660 ± 10 nm which is of particular importance for photodynamic therapy. However, the extinction coefficient for haematoporphyrins is relatively low. Hence a higher concentration of this sensitizer has to be introduced into the cancer cell for a successful photodynamic

therapy. Since some of the porphyrins are phototoxic, the advantage of chlorin E₆ is that it is rapidly eliminated after administration. Only 4 to 6 % of the administered amount is still detectable in the human body 24 hours after administration.

However, chlorin E₆ and its salts are relatively unstable in solution as well as in a lyophilized state at room temperature.

The complex described above of chlorin E₆ with polyvinylpyrrolidone in the stated composition has a considerably improved stability and therefore allows a considerably better application in practice. However, its accumulation in cancerous tissue compared to healthy tissue is still not quite satisfactory.

Hence the object of the invention is to eliminate the above disadvantages and to provide an agent based on chlorin E₆ for photodynamic therapy which not only has a stability that is well suited for handling but also has a substantially improved accumulation factor in cancerous tissue compared to healthy tissue. Another object of the invention is to provide further medical uses of chlorin E₆.

This object is achieved according to the invention by an agent for photodynamic therapy based on chlorin E₆ and its derivatives, and polyvinylpyrrolidone, which is characterized in that it contains or is composed of chlorin E₆ and polyvinylpyrrolidone in a weight ratio of 1: (> 1.5). A preferred weight ratio of chlorin E₆ to polyvinylpyrrolidone is 1: (> 1.5 to 25), preferably 1:2, more preferably 1:3, more preferably 1:5, even more preferably 1:10, even more preferably 1:15 and even more preferably 1:25.

Hence the weight ratio is preferably in the range of about 1: (5 to 25), preferably 1: (10 to 25), more preferably 1: (15 to 25), and more preferably 1: (15 to 20). The chlorin E₆ and the polyvinylpyrrolidone are preferably present as a complex.

Surprisingly, an agent having the composition stated above enables of chlorin E₆ to be accumulated in cancerous tissue compared to healthy tissue with a several-fold higher selectivity. This substantially increased accumulation not only allows the use of a lower dose of the agent but also increases the effectiveness of the laser treatment at greater depths which can be increased from previously about 1 cm penetration depth through the skin into the tissue to twice this depth.

In order to prepare the agent according to the invention, polyvinylpyrrolidone is appropriately dissolved in an aqueous base suitable for injection and then chlorin E₆ is added while continuously stirring in the amount required to achieve the desired composition of more than 1.5 and up to 25 parts by weight PVP to 1 part by weight chlorin E₆ and stirred until a completely homogenous mixture has formed. The solution obtained can be sterilized by filtration and can be freeze-dried and stored in this form at normal temperature. The formulation can also be prepared in such a manner that it is suitable for a systemic and/or local action by parenteral, enteral and/or topical administration.

The superior effectiveness of the agent according to the invention is surprising since the above-mentioned Russian patent expressly cautions against using more than 60 % by weight polyvinylpyrrolidone since excess amounts no longer react with chlorin E₆ and represent unnecessary ballast. This prejudice is overcome by the invention and a several-fold higher accumulation is achieved without reducing the stability of the complex.

A preparation of 6 to 12 kDa is preferred as the polyvinylpyrrolidone.

A complete tumour necrosis up to a depth of 20 mm was observed in animal experiments at a dose of the agent according to the invention of 1 to 5 mg/kg and laser irradiation at a wavelength of 660 nm and an energy exposition of 50 J/cm². Under otherwise identical conditions necroses were observed with the known

complex only up to a depth of 16 mm, a corresponding dose of chlorin E₆ alone only resulted in a partial tumour necrosis of up to 7 mm depth. The toxicity measured as the LD₅₀ was determined to be less than 140 mg/kg. Chlorin E₆ is appropriately used in the form of its alkali salt. Derivatives of chlorin E₆ (13-carboxy-17-[2-carboxyethyl]-15-carboxymethyl-17,18-transdihydro-3-vinyl-8-ethyl-2,7,12,18-tetramethylporphin) such as the corresponding 15-carboxyethoxymethyl or 15-formyl compounds which all occur naturally as accompanying substances of chlorin E₆ are suitable in the same way. In particular the complex according to the invention can also contain mixtures of chlorin E₆ with its derivatives.

The agent according to the invention is usually administered in the form of an injectable solution. However, it is also possible to incorporate it into ointments or liniments for direct application on the skin. An amount of 0.5 to 10 mg/kg, preferably 1 to 7 mg/kg is recommended as the dosage.

It is also possible to prepare the agent according to the invention in liquid or semi-solid pharmaceutical formulations. Formulations for topical, intravenous and/or systemic administration and in particular for a systemic and/or local action are particularly preferred.

In connection with the present invention it was also found that chlorin E₆ is suitable for preparing pharmaceutical preparations for other applications than for tumour diseases.

Surprisingly, it turned out that chlorin E₆ is very effective on the skin so that skin diseases can be readily treated with agents containing chlorin E₆ and PVP as preventive measures and also for treatment. In particular, chlorin E₆-PVP is effective against fungal diseases as well as psoriasis and similar skin diseases. It is effective against dermatophytes, moulds and yeasts.

Furthermore it has also turned out that chlorin E₆ is surprisingly suitable for epilation i.e. for hair removal.

Hence another subject matter of the present invention is an agent for the prophylactic or therapeutic or cosmetic treatment of the skin, especially for treating fungal diseases of the skin, psoriasis or for hair removal, wherein the agent comprises chlorin E₆ and PVP in any mixing ratio. The weight ratios of the two components can comprise an excess of chlorin E₆ or also an excess of PVP. In particular weight ratios of chlorin E₆ to PVP of about 1 : 0.1 up to a considerable excess of polyvinylpyrrolidone compared to chlorin E₆ and in particular up to 1 : 25 are suitable. Weight ratios of 1 : 1 are particularly preferred. However, ratios of 1 : (\geq 1.5), 1 : 5 or 1 : 10 or 1 : 15 are also suitable for the said applications.

The following examples illustrate the effectiveness of the agent according to the invention compared to a known agent according to the Russian Patent No. 2152790 with regard to tumour effectiveness and the effectiveness of chlorin E₆ for treating the skin.

Example 1

An agent referred to as Fotolon according to the Russian Patent No. 2152790 having the composition chlorin E₆ : polyvinylpyrrolidone in a weight ratio of 1 : 1 and an agent according to the invention having the composition chlorin E₆ : polyvinylpyrrolidone in a weight ratio of 1 : 10 were examined.

The investigations were carried out on 12 white raceless rats weighing between 150 and 180 g with an intraabdominally transplanted Pliss lymphosarcoma. On the 5th day after tumour transplantation all 4 groups of animals (3 rats in each group per preparation) were intravenously administered Fotolon or the agent according to the invention in a dose of 5.0 mg/kg body weight.

The accumulation dynamics of Fotolon or the agent according to the invention were observed in the tumour tissues of the rats (Pliss lymphosarcoma) and the healthy tissues (in the skin on the opposite side of the thigh) with the aid of computer-controlled fluorescence spectrophotometry using the analyser "LESA-6" (diagnostic laser "LGH 633-25" (figure 1)).

The measurements were carried out each hour during the 8 hours after administration of the preparations and after 24 hours.

The individual and average accumulation data of the preparation in the 12 rats are shown in tables 1 to 4.

Table 1: Accumulation dynamics of Fotolon in healthy tissues of rats with Pliss lymphosarcoma

| Time | 1h | 2h | 3h | 4h | 5h | 6h | 7h | 8h | 24h |
|---------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| No. 1 | 372 | 913 | 1067 | 996 | 889 | 823 | 829 | 760 | 495 |
| | 423 | 839 | 929 | 992 | 880 | 817 | 858 | 758 | 473 |
| | 380 | 806 | 852 | 939 | 907 | 835 | 855 | 766 | 511 |
| | 391 | 902 | 936 | 1004 | 898 | 829 | 832 | 773 | 507 |
| | 536 | 897 | 934 | 965 | 876 | 836 | 806 | 797 | 489 |
| No. 2 | 411 | 526 | 699 | 662 | 663 | 616 | 637 | 563 | 512 |
| | 380 | 588 | 712 | 628 | 685 | 683 | 631 | 608 | 587 |
| | 372 | 620 | 734 | 770 | 714 | 658 | 659 | 671 | 560 |
| | 498 | 576 | 714 | 691 | 699 | 689 | 661 | 594 | 545 |
| | 546 | 639 | 687 | 743 | 684 | 611 | 621 | 562 | 590 |
| No. 3 | 506 | 782 | 811 | 843 | 775 | 755 | 709 | 712 | 534 |
| | 428 | 689 | 756 | 767 | 680 | 631 | 642 | 584 | 525 |
| | 398 | 652 | 721 | 735 | 669 | 667 | 653 | 611 | 536 |
| | 411 | 793 | 811 | 892 | 721 | 688 | 696 | 646 | 587 |
| | 402 | 746 | 809 | 857 | 704 | 691 | 678 | 623 | 544 |
| $\bar{x} \pm$ | 430.3 | 731.2 | 811.5 | 832.3 | 762.9 | 721.8 | 717.8 | 668.5 | 533.1 |
| Sx | 15.6 | 33.03 | 28.9 | 33.2 | 24.9 | 30.0 | 23.3 | 21.8 | 9.5 |

Table 2: Accumulation dynamics of Fotolon in tumour tissue of rats with Pliss lymphosarcoma

| Time | 1h | 2h | 3h | 4h | 5h | 6h | 7h | 8h | 24h |
|-------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| No. 1 | 1428 | 2422 | 3690 | 4030 | 3646 | 3178 | 2470 | 2255 | 1465 |
| | 1318 | 2359 | 3448 | 4209 | 3212 | 3017 | 2375 | 2214 | 1403 |
| | 1351 | 2218 | 3528 | 4028 | 3106 | 2990 | 2242 | 2205 | 1492 |
| | 1557 | 2330 | 3466 | 4250 | 3624 | 3105 | 2386 | 2101 | 1468 |
| | 1458 | 2221 | 3322 | 4194 | 3514 | 2998 | 2237 | 2138 | 1489 |
| No. 2 | 1066 | 1561 | 2533 | 2895 | 3324 | 2810 | 2213 | 2111 | 1564 |
| | 1077 | 1694 | 2538 | 2951 | 3291 | 2888 | 2115 | 2016 | 1684 |
| | 1044 | 1850 | 2771 | 3083 | 3278 | 2967 | 2253 | 2117 | 1614 |
| | 1121 | 1993 | 2649 | 2825 | 3127 | 2930 | 2178 | 2005 | 1588 |
| | 1096 | 1843 | 2651 | 3136 | 3184 | 2803 | 2169 | 2104 | 1560 |
| No. 3 | 1223 | 2113 | 3246 | 3784 | 3113 | 2835 | 2340 | 2111 | 1623 |
| | 1325 | 2235 | 3126 | 3630 | 3087 | 2769 | 2254 | 2147 | 1648 |
| | 1267 | 2156 | 3090 | 3475 | 2970 | 2834 | 2365 | 2138 | 1705 |
| | 1284 | 2173 | 3117 | 3657 | 2785 | 2812 | 2411 | 2119 | 1655 |
| | 1311 | 2328 | 3215 | 3712 | 3116 | 2809 | 2389 | 2108 | 1589 |
| x± | 1261.7 | 2099.7 | 3092.7 | 3590.6 | 3225.1 | 2916.3 | 2293.1 | 2125.9 | 1569.8 |
| Sx | 40.2 | 66.4 | 98.1 | 130.6 | 60.3 | 31.7 | 26.9 | 16.9 | 23.1 |

Table 3: Accumulation dynamics of the agent according to the invention in healthy tissues of rats with Pliss lymphosarcoma

| Time | 1h | 2h | 3h | 4h | 5h | 6h | 7h | 8h | 24h |
|-------|-------|-------|--------|--------|--------|--------|--------|-------|-------|
| No. 1 | 666 | 744 | 988 | 1105 | 1120 | 1141 | 1045 | 911 | 712 |
| | 673 | 735 | 963 | 1138 | 1163 | 1108 | 997 | 932 | 795 |
| | 636 | 721 | 1012 | 1120 | 1102 | 1128 | 1102 | 914 | 773 |
| | 612 | 691 | 996 | 1163 | 1109 | 1120 | 1008 | 930 | 689 |
| | 644 | 696 | 925 | 1132 | 1195 | 1137 | 989 | 911 | 704 |
| No. 2 | 788 | 813 | 1131 | 1231 | 1247 | 1174 | 1115 | 1076 | 811 |
| | 749 | 829 | 1115 | 1215 | 1275 | 1182 | 1095 | 938 | 773 |
| | 805 | 825 | 1109 | 1209 | 1252 | 1246 | 1117 | 990 | 735 |
| | 731 | 818 | 1098 | 1298 | 1307 | 1214 | 1003 | 895 | 791 |
| | 707 | 793 | 1150 | 1250 | 1258 | 1185 | 990 | 978 | 806 |
| No. 3 | 698 | 788 | 946 | 1121 | 1137 | 1210 | 1118 | 1026 | 921 |
| | 712 | 813 | 938 | 1132 | 1182 | 1289 | 1045 | 966 | 885 |
| | 722 | 768 | 980 | 1098 | 1148 | 1303 | 1132 | 992 | 830 |
| | 741 | 824 | 1034 | 1117 | 1163 | 1241 | 1067 | 969 | 813 |
| | 708 | 775 | 1057 | 1134 | 1190 | 1266 | 1054 | 973 | 867 |
| x± | 706.1 | 775.5 | 1029.5 | 1164.2 | 1189.9 | 1196.3 | 1058.5 | 960.1 | 793.7 |
| Sx | 13.9 | 12.3 | 19.5 | 15.7 | 16.6 | 16.3 | 13.5 | 12.7 | 17.2 |

Table 4: Accumulation dynamics of the agent according to the invention in tumour tissues of rats with Pliss lymphosarcoma

| Time | 1h | 2h | 3h | 4h | 5h | 6h | 7h | 8h | 24h |
|-------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| No. 1 | 2453 | 3776 | 6898 | 7683 | 8934 | 9115 | 7770 | 6235 | 3501 |
| | 2378 | 3930 | 6979 | 7326 | 8930 | 9087 | 6823 | 5167 | 3639 |
| | 2346 | 3979 | 6815 | 7435 | 8805 | 8992 | 7590 | 5280 | 3603 |
| | 2482 | 3831 | 6723 | 7762 | 8980 | 9046 | 6805 | 5330 | 3654 |
| | 2398 | 3678 | 6516 | 7996 | 8902 | 9087 | 7672 | 4289 | 3625 |
| No. 2 | 2662 | 3890 | 6930 | 8096 | 9112 | 9207 | 7012 | 5654 | 3701 |
| | 2696 | 4012 | 6914 | 8135 | 9289 | 9246 | 7046 | 5720 | 3790 |
| | 2524 | 3965 | 6918 | 8248 | 9293 | 9301 | 7994 | 5612 | 3794 |
| | 2483 | 3894 | 6896 | 8113 | 9307 | 9412 | 7023 | 5750 | 3845 |
| | 2708 | 3979 | 6727 | 8260 | 9315 | 9385 | 7110 | 5711 | 3810 |
| No. 3 | 2776 | 4112 | 7313 | 9080 | 9224 | 9305 | 7087 | 4693 | 4025 |
| | 2730 | 4102 | 7222 | 9217 | 9336 | 9378 | 7116 | 5166 | 3986 |
| | 2560 | 4137 | 7290 | 9112 | 9217 | 9402 | 7023 | 5668 | 3912 |
| | 2684 | 3980 | 7572 | 9304 | 9341 | 9235 | 7114 | 5713 | 4048 |
| | 2712 | 4047 | 7284 | 9132 | 9304 | 9198 | 7200 | 4690 | 4115 |
| x± | 2572.8 | 3954.1 | 6999.8 | 8326.6 | 9151.9 | 9226.4 | 7225.7 | 5373.7 | 3802.5 |
| Sx | 37.3 | 32.8 | 72.4 | 174.1 | 49.1 | 35.6 | 91.9 | 132.3 | 47.8 |

Analysis of the results obtained confirms that a substantially improved selective storage in tumour tissue of rats is observed with the agent according to the invention – see figure 1 and 2 of the drawings.

Table 5: Coefficient of the accumulation selectivity of Fotolon

| Time | 1h | 2h | 3h | 4h | 5h | 6h | 7h | 8h | 24h |
|-------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| tumour | 1261.7 | 2099.7 | 3092.7 | 3590.6 | 3225.1 | 2916.3 | 2293.1 | 2125.9 | 1569.8 |
| thigh skin | 430.3 | 731.2 | 811.5 | 832.3 | 762.9 | 721.9 | 717.8 | 668.5 | 533.1 |
| coefficient | 2.93 | 2.87 | 3.81 | 4.31 | 4.23 | 4.04 | 3.19 | 3.18 | 2.94 |

Table 6: Coefficient of the accumulation selectivity of the agent according to the invention

| Time | 1h | 2h | 3h | 4h | 5h | 6h | 7h | 8h | 24h |
|-------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| tumour | 2572.8 | 3954.1 | 6998.8 | 8326.6 | 9151.9 | 9226.4 | 7225.7 | 5373.7 | 3802.5 |
| thigh skin | 706.1 | 775.5 | 1029.5 | 1164.2 | 1189.9 | 1196.3 | 1058.5 | 960.1 | 793.7 |
| coefficient | 3.64 | 5.23 | 6.80 | 7.15 | 7.69 | 7.71 | 6.83 | 5.60 | 4.79 |

Example 2

Tumour investigations

-preclinical investigations of Fotolon (chlorin E₆ – PVP 1:10) with regard to phototoxicity in a HET-CAM assay

The HET-CAM (chorioallantoic membrane) bioassay is important for evaluating the effects of photodynamic therapy on vessels and transplanted tumours. An advantage of the assay over animal models is that morphological changes in vessels and changes in blood perfusion/circulation can be qualitatively and quantitatively determined *in vivo* in a non-invasive manner. There are numerous experimental approaches to investigating the effect of phototoxic substances on incubated hen's eggs with regard to the site of application, the time of application and the criteria for assessing the findings and evaluation.

The CAM as part of the extraembryonic vascular system was selected as the site of application. The CAM is highly vascularized, transparent and develops very dynamically between day 3 and 12.

In the first investigations a comparison was carried out between Photosan-3 (haematoporphyrin derivative), Seehofer Laboratorium GmbH Company and Fotolon (chlorin E₆ – PVP 1:10) with regard to toxicity without irradiation, variable light dosage and variable photosensitizer concentration.

The following results have so far been found:

- in the case of Fotolon no toxicity occurs without irradiation
- a dose-dependent phototoxic reaction was found as a function of the Fotolon concentration and the light dose at the same power density.

Example 3

Investigations on extending the applications

In order to determine the improved efficacy of Fotolon (as a combination of chlorin E₆ and PVP in various compositions including 1:1, 1:10) compared to pure chlorin E₆, investigations were firstly carried out with pure chlorin E₆ without adding PVP.

These included:

1. the treatment of psoriasis
2. the treatment of human fungal diseases in vitro and in vivo (athlete's foot) and
3. the epilation of hairs.

Treatment of psoriasis:

Chlorin E₆ was applied topically in a gel on the corresponding skin areas of a patient and allowed to act for 30 min under occlusion. Irradiation was carried out with a laser at a wavelength of 662 nm for 3 to 5 minutes and a dose of 36 to 60 J/cm². A UVB irradiation (without further addition of a medicinal drug) of psoriasis-damaged skin areas was carried out as a comparison.

An initial result was that the skin areas treated with PDT healed more rapidly.

The investigations on the treatment of human fungal diseases were carried out in the following simplified manner:

A small amount of the fungal tissue is removed from patients with a fungal disease for a pathological determination. A portion of this is transferred to a nutrient medium. The resulting colonies are separated and separately subjected to a photodynamic therapy (PDT).

In order to prepare for the PDT the individual fungal colonies were overlaid with a photosensitizer solution. The concentration of the photosensitizer in the solution was varied (among others 5, 10, 20 % and without a photosensitizer solution as a control) in order to determine the concentration range in which the photosensitizer acts. After the photosensitizer solution had acted on the fungal cultures for 30 minutes, they were irradiated. Among others it was examined how often the irradiation has to be repeated and at which intensity in order to achieve an adequate effectiveness.

Up to now the following fungal species have been included in the PDT with chlorin E₆:

Moulds: *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium species*, *Mucor species*

Yeasts: *Candida albicans*, *Candida glabrata*

Dermatophytes: *Trichophyton rubrum*, *Microsporum gypseum*, *Trichophyton mentagrophytes*

The results can be interpreted as follows:

Photodynamic therapy has the largest effect in the treatment of dermatophytes. The best results were obtained with the fungal cultures **Trichophyton mentagrophytes** and **Trichophyton rubrum**. In contrast PDT had no significant effect on the fungal culture **Microsporum gypseum**.

Among the various moulds a visible effect of photodynamic therapy is observed on the fungal cultures **Penicillium species**, **Aspergillus fumigatus** and **Aspergillus niger**. In contrast the fungal species **Mucor** was sensitive to PDT.

Different results were also obtained for yeasts. PDT treatment had a major effect on **Candida albicans** (the irradiated colonies died off completely). However, the fungal culture **Candida glabrata** was insensitive to PDT until the end of treatment.

Treatment of a patient with athlete's foot (**Aspergillus fumigatus**) by chlorin E₆ and subsequent irradiation resulted in a regression of the fungus. Chlorin E₆ was incorporated into a commercial gel and applied topically. Other parameters of the treatment were: chlorin E₆ concentration = 0.2 %, a laser having an irradiation wavelength of 662 nm and a power of 200 mW was used as a light source, the number of irradiations was 6, the irradiation was repeated weekly, irradiation time: 3 minutes for the first two irradiations and 5 minutes for the subsequent four irradiations.

However, treatment of one patient does not provide reliable information on the actual effectiveness of chlorin E₆. A larger number of patients were included in the treatment with Fotolon in various compositions (1:1, 1:10).

Hair epilation:

Hair epilation was carried out on various hair areas of the patient. Chlorin E₆ was incorporated into a commercial gel to a final concentration of 0.1 % and 0.2 % and applied to the corresponding skin regions. After 30 minutes time to take effect the hairy surface was irradiated for 3 to 5 minutes with a laser at an energy dose of 36 J/cm² or 60 J/cm². The hairs were subsequently removed (razor, tweezers). The chest, groin, upper lip and lower abdomen were selected as the hair areas. Depending on the success of the treatment, the irradiation was repeated once weekly within 2 to 4 weeks.

The following results were found:

- chest: most hairs did not grow again, the hairs which did grow again are very fine
- groin: most hairs did not grow again, the hairs which did grow again are very fine
- upper lip: hairs did not grow again
- lower abdomen: hairs did not grow again

These results show the effectiveness of chlorin E₆ in the treatment of psoriasis, individual fungal diseases and epilation.